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Electrochemical behaviour of anticancer drug lomustine and in situ evaluation of its interaction with DNA

Paulina Andréa V. de Carvalho[a,b], Ilanna Campelo Lopes[b], Elizaura Hyeda C. Silva[b], Evellin Enny S. Bruzaca[b], Hugo José Alves[c], Mayara Ingrid S. Lima[c], Auro Atsushi Tanaka[b,d,*]

[a] Instituto Federal de Educação, Ciência e Tecnologia do Maranhão, CEP 65363-000, Zé Doca, MA, Brazil
[b] Departamento de Química, Centro de Ciências Exatas e Tecnologia, Universidade Federal do Maranhão, CEP 65.080-805, São Luís, MA, Brazil
[c] Departamento de Ciências Biológicas, Centro de Ciências Biolóicas e da Saúde, Universidade Federal do Maranhão, CEP 65.080-805, São Luís, MA, Brazil
[d] Instituto Nacional de Ciência e Tecnologia de Bioanalítica, Caixa Postal 6154, CEP 13083-970, Campinas, SP, Brazil

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ABSTRACT

Electrochemical techniques were used to investigate the behavior of lomustine (CCNU) and its degradation in aqueous solution at a glassy carbon electrode (GCE). The in situ interaction of CCNU and chemically degraded CCNU (cdCCNU) with dsDNA was then investigated in dsDNA incubated solutions, using dsDNA electrochemical biosensors and comet assays. CCNU undergoes electrochemical reduction in two irreversible, diffusion-controlled, and pH-dependent redox processes, each with transfer of two electrons and one proton. At pH ≥ 10.1, the peak potential for the two processes was essentially pH-independent and involved only one electron. A mechanism was proposed for the reduction of CCNU in a neutral medium. In addition, it was found that CCNU underwent spontaneous degradation during incubation in aqueous solution, without the formation of electroactive degradation products. The degradation process was faster in basic media. Moreover, this pro-drug interacted with the DNA. Its metabolite(s) initially caused condensation of the double helix chains, followed by the unwinding of these chains. In addition, free guanine (Gua) was released from the dsDNA and oxidative damage to the DNA by the CCNU metabolite(s) was evidenced from the detection of 8-oxoGua and 2,8-oxoAde. These results were confirmed by the poly(dA)- and poly(dG)-polyhomonucleotide biosensors, which revealed the oxidative damage caused to both bases (guanine and adenine) of the dsDNA by the CCNU metabolite(s). The comet assay indicated breaks in the single strand DNA, complementing the results of the studies using differential pulse voltammetry. Conformational changes of dsDNA caused by CCNU and cdCCNU were confirmed using comet assays.

1. Introduction

Lomustine (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea) is a bifunctional alkylating antineoplastic of the nitrosurea class, which is used primarily in the treatment of brain tumors [1] (Scheme 1). In solution at physiological pH, the compound spontaneously degrades to produce the main metabolite, the 2-chloroethyl carbon ion, which causes alkylation of the guanine and adenine bases of DNA. Displacement of the chlorine atom can result in intra- or inter-strand cross-linking of the DNA, with formation of monoadducts that consequently prevent DNA replication. The spontaneous degradation of CCNU can produce isocyanates capable of carbamylating protein lysine residues, and this reaction may inactivate certain DNA repair enzymes [2].

The mechanisms of drug interactions with DNA have been extensively studied during the last decades [3–6], with these interactions having a close relationship with the carcinogenic, toxicological, or pharmacological activities of the substances tested [7]. The interactions between DNA and chemical species including metal ions and their complexes, water, organic molecules, and proteins are reversible. In addition, such interactions can stabilize DNA conformation. On the other hand, the DNA interactions of compounds including carcinogenic and pharmacological drugs can result in irreversible damage. Detailed investigation of such interactions is essential in order to provide information concern-

* Corresponding author at: Departamento de Química, Centro de Ciências Exatas e Tecnologia, Universidade Federal do Maranhão, 65080-805, São Luís MA Brazil
E-mail address: tanaka.auro@ufma.br (A.A. Tanaka).

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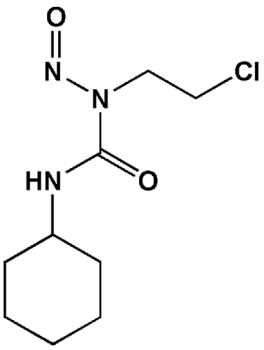


Scheme 1. Chemical structure of CCNU.

ing harmful substances and assist in the development of new chemotherapeutics [7].

The main methods reported in the literature for the detection, characterization, and determination of CCNU and its degradation products, as well as for investigation of the interaction of this drug with DNA, are spectrometric and chromatographic techniques [8–11]. Alternatively, electrochemical methods have attracted attention due to their sensitivity, selectivity, simplicity, moderate cost, and capacity for miniaturization [12]. Studies of oxidation and reduction processes in various media, surface adsorption, and electron transfer mechanisms, including the use of modified electrodes, provide examples of some of the many current applications of these methods [13]. In addition, such techniques can be used to investigate the mechanisms and dynamics of the interactions between DNA and drugs [3,7,14].

By observing the electrochemical signals of the interactions of drugs with DNA, it is possible to elucidate the mechanisms of interaction, the conformation of adducts, the nature of the complexes formed, and the presence of constant bonds [15]. There is considerable current interest in the development of DNA-based electrochemical biosensors able to provide rapid and cheap diagnosis of genetic diseases, as well as for other applications [16]. Compared to other methods, these sensors offer high sensitivity in the detection of small perturbations in the double helix structure [17], as well as in investigation of the mechanisms of oxidative damage caused to DNA.

In this work, investigation was made of the electrochemical behavior of CCNU in an aqueous medium and its chemical degradation on a glassy carbon electrode, as well as the in situ interaction of this pro-drug with DNA, using electrochemical biosensors based on DNA structures. The objective was to obtain complementary information about the compound studied, as well as to show that the methodology used is simple, accessible, effective, and could contribute to studies of new anticancer drugs.

2. Experimental

2.1. Reagents and solutions

The CCNU (≥ 98% purity) and dsDNA (deoxyribonucleic acid sodium salt from calf thymus) were obtained from Sigma. Stock solutions of 1.00 mmol L^{-1} CCNU in ethanol:water (2:1) were prepared and stored at 4 °C until use. Electrolyte solutions were prepared at different pH values, using analytical grade reagents and purified water obtained from a Milli-Q Direct 8 system (Millipore). The compositions and corresponding pH values of the electrolyte solutions were as follows: acetate buffer (pH 3.6-5.3), phosphate buffer (pH 6.1-8.0), $Na_2B_4O_7.10H_2O$/NaOH (pH 9.2-10.1), and NaOH/KCl (pH 11.1-12.0) [18]. The pH of the solutions was measured with a Metrohm 827 pH meter.

Solutions of dsDNA were prepared by dissolving ~1.5 mg of dsDNA in 5 mL of purified water and keeping at 4 °C for 24 h for complete homogenization. Subsequently, its concentration was determined by UV-Vis spectroscopy, with multiplication of the absorbance obtained by the conversion factor (1 u $A_{260\ nm}$ =50 μg mL^{-1} of dsDNA) [19].

In order to avoid the presence of oxygen, the solutions were saturated with N_2 gas (White Martins) for 10 min prior to each electrochemical measurement in the cathodic region, and a flow of N_2 was maintained above the solution during the potential sweeps.

2.2. Voltammetric measurements

The electrochemical measurements were performed with an Autolab PGSTAT 302 N potentiostat and an electrochemical system (acquired from DAQ Inc.) composed of a glass cell (~2 mL solution capacity), a glassy carbon electrode (GCE, Φ =1.0 mm) as the working electrode, an Ag/AgCl electrode (Φ =2.0 mm) as the reference electrode, and a coiled platinum wire (Φ =0.5 mm) as the auxiliary electrode. The cyclic voltammograms were recorded using a scan rate (v) of 50 mV s^{-1} and potential step (ΔE) of 2 mV. The differential pulse voltammetry measurements employed a pulse width (ΔEs) of 70 ms, pulse amplitude (ΔEp) of 50 mV, and potential scan rate of 5 mV s^{-1}. Square wave voltammetry experiments were performed with ΔEs of 2 mV, ΔEp of 50 mV, and frequency (f) of 50 Hz.

2.3. Data acquisition and presentation

The differential pulse voltammograms were corrected using a moving average function with a 2 mV step window, employing GPES v. 4.9 software. This mathematical treatment improved the definition and identification of the peaks, relative to the baseline, without the introduction of any artifacts, although in some cases the peak current was reduced (≤ 10%) compared to the untreated voltammetric curve. The values of the peak currents were obtained from the original voltammograms, using only baseline correction.

2.4. Pretreatment of the GCE surface

Before each measurement, the GCE was pretreated by polishing the surface on filter paper wetted with a diamond spray (1 μm particle size, Kemet International Ltd., UK), followed by cleaning with deionized water to remove possible adsorbed residues. Electrochemical conditioning of the GCE surface was then carried out with successive voltammetric scans in the supporting electrolyte, in order to achieve a stable baseline prior to use of the electrode in each technique.

2.5. CCNU chemical degradation

Solutions of CCNU at a concentration of 0.5 mmol L^{-1} in supporting electrolytes with different pH values (from 3.6 to 12.0), used in the initial experiments, were incubated for different times (0 h, 24 h, and 48 h) and were investigated by differential pulse voltammetry.

For verification of the voltammetric results, analyses were also performed using high performance liquid chromatography (HPLC). In the degradation study of 0.5 mmol L^{-1} CCNU in acetate buffer at pH 3.0, analysis by HPLC-DAD (Model SPD-M20A, Shimadzu) was performed using mobile phase A composed of a mixture of phosphate buffer and acetonitrile (80:20, v/v), and mobile phase B composed of phosphate buffer and acetonitrile (24:76, v/v). A Shimadzu C18 column was kept at a temperature of 40 °C, the injection volume was 20 μL, and the detector wavelength was 254 nm.

2.6. Interaction study

2.6.1. Procedure 1: Interaction of CCNU with DNA in solution

Stock solutions of dsDNA in water were prepared weekly and maintained at 4 °C. Aliquots of these solutions were diluted to a concentration of 100 μg mL^{-1} in acetate buffer (pH 4.5) and were incubated with 0.1 mmol L^{-1} of freshly prepared CCNU solution and with solutions that had been chemically degraded (cdCCNU) for times of 10 min, 1 h, 2.5 h, 3.5 h, 24 h, and 48 h. As controls, dsDNA solutions were prepared in pH 4.5 acetate buffer under the same conditions described above, in the absence of CCNU (or cdCCNU).

2.6.2. Procedure 2: Interaction of CCNU with the DNA biosensor

Films of dsDNA, poly(dA), and poly(dG) were immobilized separately on the surface of the clean GCE conditioned in acetate buffer (pH 4.5), with deposition of 5 μL of 100 μg mL^{-1} of each solution, diluted in acetate buffer (pH 4.5). After drying the first layer, the procedure was repeated two more times. The biosensors were then rinsed with deionized water to remove possible residues of DNA structures and were immediately immersed in either freshly prepared 0.5 mmol L^{-1} CCNU solution or in the solution of cdCCNU degraded during 14 days. The biosensors were left under incubation in these solutions for times of 10 min, 1 h, 2 h, 24 h, 48 h, and 72 h.

In parallel, control experiments were performed with the dsDNA biosensors, following the same steps described above and with the same incubation times, but in the absence of CCNU and degraded CCNU. These experiments were performed to evaluate the stability of the dsDNA layers immobilized on the glassy carbon surface, after the incubation time. The voltammograms revealed no changes in the double helix structure. The same procedures used for the poly(dA) and poly(dG) biosensors were also used in the study of the guanosine (dGuo) and adenosine (dAdo) nucleotides in the absence of CCNU and degraded CCNU. Once again, the voltammograms showed that there were no changes of these polyhomonucleotides.

2.7. Comet assays

For the in vitro comet assays were used human peripheral blood mononuclear cell (PBMCs) in culture. The procedures for obtaining the mononuclear cell samples and for the comet tests were performed according to the literature [18]. The lymphocytes were treated with 0.5 mmol L^{-1} CCNU for 3 h and 24 h, and the results were compared with a negative control without CCNU and with a positive control containing H_2O_2 (20%). Aliquots of 10 μL of the samples were mixed with low melting point agarose (0.5%, m/v) and the mixtures were dripped onto slides with agarose. The material was refrigerated at 4 °C for 5 min, followed by denaturation in pH 10.0 lysis solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris, 1% Triton X-100, and 10% DMSO) and subsequent refrigeration for 24 h. The slides were then immersed in alkaline solution (pH 13 TAE buffer with 10 mol L^{-1} NaOH and 0.2 mol L^{-1} EDTA) and incubated for 20 min at 4 °C. Electrophoresis was performed at 25 V (300 mA) for 20 min, followed by neutralization with 0.4 mol L^{-1} Tris-HCl solution (pH 7.5). The slides were fixed in 100% ethanol, dried, and stained with ethidium bromide. The analyses were performed using a fluorescence microscope (Model BX61, Olympus), with 100 cells being counted at random.

2.7.1. Statistical analysis

The data were analyzed for normality using the Shapiro-Wilk test. The results of the comet tests were evaluated using the one-way ANOVA parametric test followed by the Dunn test for comparison of two groups simultaneously. The analyses were performed using GraphPad Prism 5.0 software, considering significant differences for $p < 0.05$.

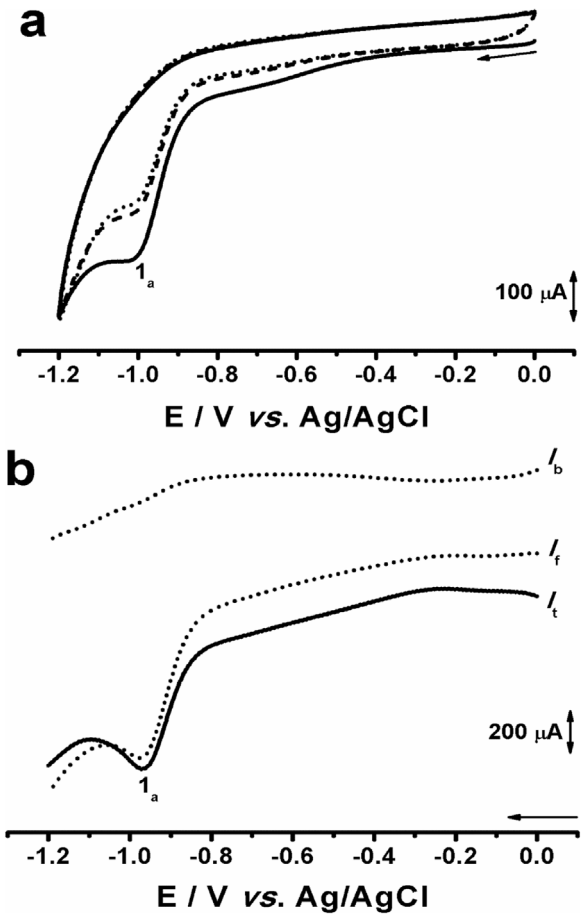


Fig. 1. a) Cyclic voltammograms for 0.8 mmol L^{-1} CCNU in 0.1 M phosphate buffer at pH 7.1: (▬) first, (▪▪▪) second, and (•••) third scans immediately after addition of CCNU to the buffer; ν =50 mV s^{-1}. b) SWV using 0.8 mmol L^{-1} CCNU in 0.1 M phosphate buffer at pH 7.1, immediately after addition of CCNU: (▬) first scan after addition of CCNU to the buffer; ν_{eff} =50 mV s^{-1}; I_t – total, I_f – forward, and I_b – backward currents.

3. Results and Discussion

3.1. Electrochemical behavior of CCNU

3.1.1. Cyclic voltammetry

The voltammograms were obtained using 0.8 mmol L^{-1} CCNU solution (phosphate buffer, pH 7.1), with an initial potential (E_i) of + 0.00 V and scanning up to inversion potential (E_{inv}) values of - 1.20 V and + 1.30 V in successive sweeps. The appearance of a new cathodic peak at E_{pc} = - 1.00 V (peak 1a, Fig. 1a) was indicative of an irreversible redox process, in agreement with previous work using a mercury film for determination of CCNU in biological fluids [12].

The influence of the scan rate on the peak current was studied under the same conditions of concentration and initial pH. The rates investigated were 150 and 450 mV s^{-1}, which revealed a shift of peak 1a to more negative potentials and an increase of the capacitive current.

The modulus value of the difference between the peak potential, E_{pc}, and the half-height potential, $E_{p1/2c}$, found experimentally was $|E_{pc} - E_{p1/2c}|$ =41.4 mV. For irreversible systems, $|E_{pc} - E_{p1/2c}|$ =47.7 mV / (αc n'), where αc is the cathodic charge transfer coefficient and n' is the number of electrons involved in the rate determining step [20], for which the αc n' value was 1.15.

With increasing scan rate, the peak current increased proportionally with the square root of the scan rate, showing that the reduction process was controlled by diffusion of the species in solution. For diffusion-driven irreversible systems, the peak current, I_{pc}, is obtained using the following equation:

$$I_{pc}(A) = -2.99x10^5\, n\left(\alpha_c n'\right) A[C]_\infty D^{1/2}\, \nu^{1/2} \quad (1)$$

where n is the number of electrons transferred during the CCNU reduction process ($n = 2$), obtained from the DPV data; A is the electroactive area of the GCE, in cm^2 ($A = 1.45 \times 10^{-3}\ cm^2$); C is the concentration of CCNU, in mol cm^{-3} ($C = 8.0 \times 10^{-7}$ mol cm^{-3}); D is the diffusion coefficient of the CCNU, in $cm^2\ s^{-1}$; and ν is the potential scan rate, in V s^{-1}. The coefficient of the linear relationship between I_{pc} and $\nu^{1/2}$ was - 2.067×10^{-6} A/(V s^{-1}), so the value of D_{CCNU} (at pH 7.1) was $7.73 \times 10^{-6}\ cm^2\ s^{-1}$. The Randles and Sevick equation [20] was used to determine the electroactive area of the GCE from the data obtained in the scan rate studies using CV with a solution of 1.0 mmol L^{-1} $K_4[Fe(CN)_6]$ in phosphate buffer (pH 7.1). The value of D adopted for $K_4[Fe(CN)_6]$ was $7.35 \times 10^{-6}\ cm^2\ s^{-1}$ [21].

3.1.2. Square wave voltammetry

The electrochemical behavior of the CCNU was also studied by square wave voltammetry. The voltammogram obtained from the first scan in a 0.8 mmol L^{-1} CCNU solution in phosphate buffer (pH 7.1) showed the reduction peak 1a at E_{pc} = - 0.97 V (Fig. 1b). The direct, reverse, and total current components confirmed the irreversibility of the reaction concerning peak 1a, since the direct current showed a similar intensity as the total current, while no reduction peak was observed for the reverse current, which confirmed that the process was irreversible.

3.1.3. Differential pulse voltammetry

The effect of pH on the reduction of CCNU was studied by DPV in the pH range from 3.6 to 12.0. The data were obtained using solutions of 0.8 mmol L^{-1} CCNU in different electrolytes (Fig. 2).

The voltammograms obtained for the first scans at the different pH values showed a cathodic peak 1a related to the reduction of CCNU on the GCE surface (Fig. 2a). As the pH was increased, peak 1a shifted towards potential that was more negative. The ratio was linear and could be described by the equation: Ep (V) = - 0.78 - 0.024 pH (Fig. 2b). The straight line slope of 24 mV/pH was close to the theoretical value of 29 mV/pH, indicating that the number of electrons was double the number of protons [12,22]. As the peak width at half height ($W_{1/2}$) of peak 1a was $\tilde{6}0$ mV, for all the pH values studied, the electrochemical reaction involved the transfer of two electrons and one proton in the rate-determining step, and could be described by the following equation [23]:

$W_{1/2} = 3.52RT/nF$ (Equation 2)

These results were similar to the previously reported finding that two electrons and one proton were transferred in the CCNU reduction mechanism [12].

At pH values of 4.3, 5.3, 9.0, and 12.0, a new peak 2a was recorded (Fig. 2a). This peak also shifted towards potential that was more negative as the pH was increased. The ratio was linear and could be described by the equation: Ep (V) = - 0.91 - 0.022 pH (Fig. 2b). The slope of the line, 22 mV/pH, indicated that the number of electrons was double the number of protons involved in the reduction mechanism of peak 2a. Considering the peak width at half height ($W_{1/2}$) of $\tilde{3}8$ mV for peak 2a, for all the pH values, the process involved two electrons and one proton. A pK_a value of $\tilde{1}0.1$ was obtained for CCNU.

3.1.4. Reduction mechanism of CCNU

The process of reduction of CCNU (peak 1a) was irreversible, pH-dependent up to pH 10.1, and involved the transfer of two electrons and one proton, leading to the formation of a reduction product (Scheme 2).

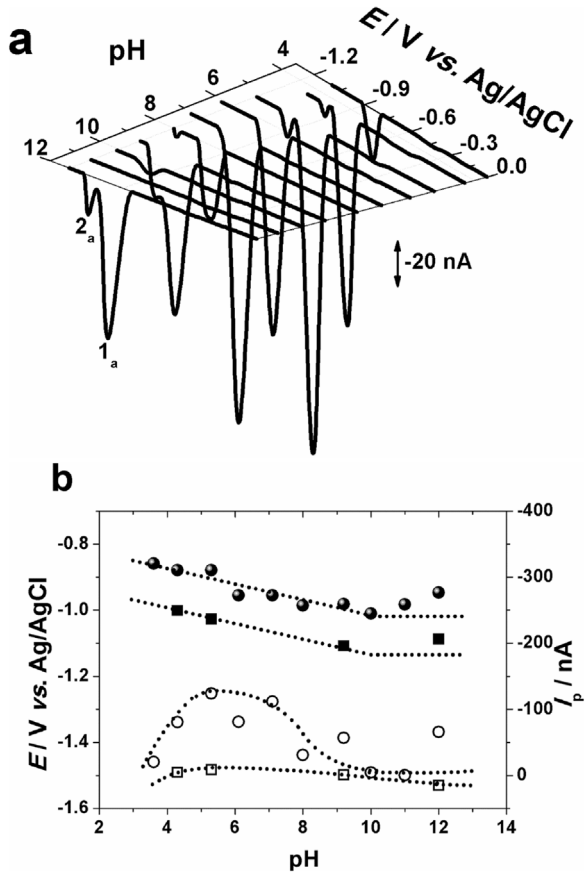


Fig. 2. Background-corrected DP voltammograms obtained immediately after the addition of 0.8 mmol L^{-1} CCNU to the supporting electrolyte, as a function of pH: a) 3D plot of first scan; b) Plots of (●) E_{p1a}, (■) E_{p2a}, (○) I_{p1a}, and (□) I_{p2a} peaks 1a and 2a, according to pH.

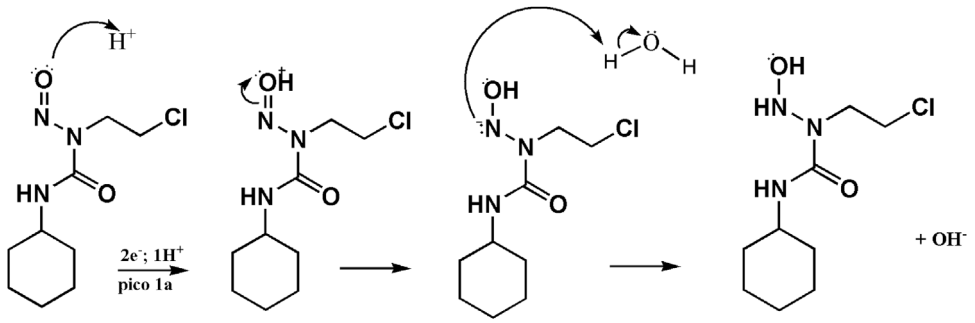
At pH 7.0, the reduction of CCNU occurs in the nitrous group present in the molecule, since it is a group more susceptible to the removal of electrons, producing a carbanion. The stabilization of this anion occurs through the nucleophilic addition of water. This results in a hydroxylamine derivative, according to a previously published study [12].

3.2. Electrochemical behavior of degraded CCNU

3.2.1. Differential pulse voltammetry

The CCNU degradation behavior was investigated at a concentration of 0.8 mmol L^{-1}. The voltammograms obtained in acetate buffer (pH 5.3) showed that after 24 h and 48 h of incubation there were successive decays of peak currents 1a and 2a (Fig. 3a). This effect was due to the spontaneous degradation of CCNU in aqueous solution, with increasing incubation time. A study carried out in the early 1990s showed that CCNU degrades spontaneously in an aqueous medium [24]. This same behavior was observed in all the supporting electrolytes with different pH values studied (Figs. 3b and c).

The decrease of peak current 1a with increasing incubation time was more accentuated in a basic medium (Fig. 4), demonstrating that CCNU tended to degrade faster in basic electrolytes [24].



Scheme 2. Proposed mechanism for reduction of 0.8 mmol L^{-1} CCNU in aqueous solution at pH 7.1.

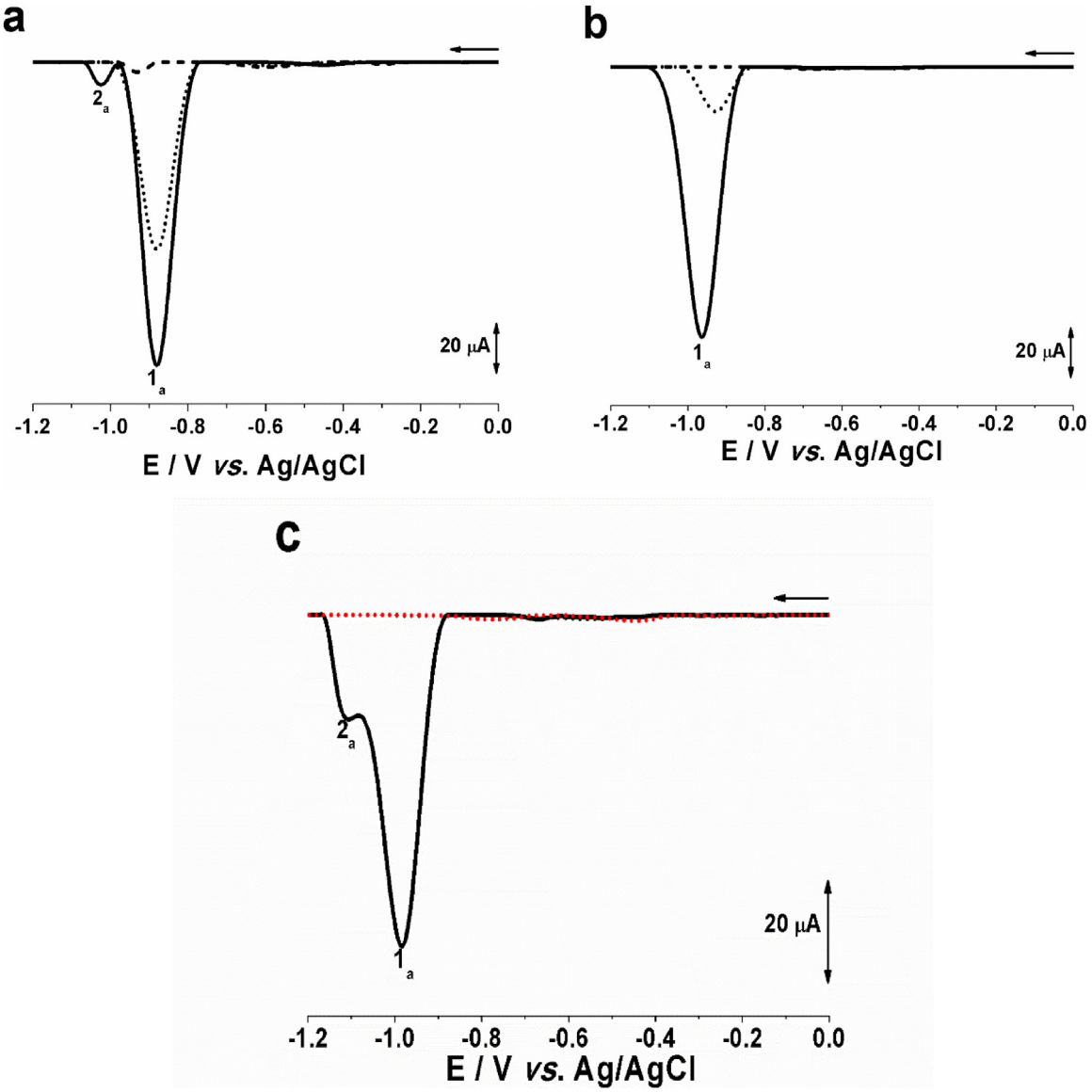


Fig. 3. Background-corrected DP voltammograms for 0.8 mmol L^{-1} CCNU in 0.1 M buffer: (a) pH 5.3, (b) pH 7.1, and (c) pH 9.2: First scan after (▬) 0 h, (•••) 24 h, and (▪▪▪) 48 h of incubation.

3.2.2. Chromatographic analysis

The evaluation of CCNU degradation was also performed using chromatographic analysis, in order to verify the results obtained electrochemically.

In the chromatogram obtained for the freshly prepared CCNU solution, only one CCNU peak was observed, with a retention time of 18 min (Fig. 5), which was close to the value reported in the literature [8]. After 24 h of incubation, the intensity of the CCNU peak decreased and two new peaks appeared, with retention times of 3.594 and 4.586 min, indicative of degradation of the pro-drug molecules in aqueous solution, with the formation of two degradation products. After 48 h, the CCNU peak decreased significantly,

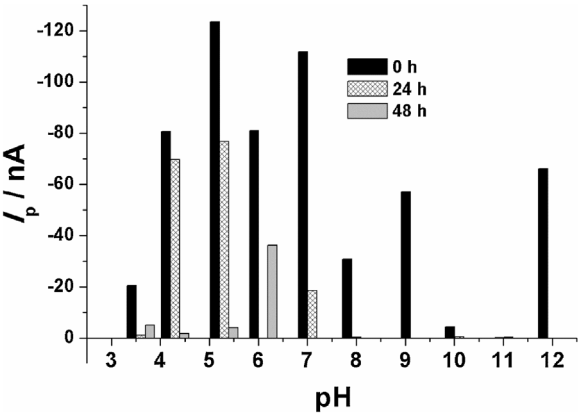


Fig. 4. Variation of the peak 1a current for 0 h, 24 h, and 48 h of incubation in the supporting electrolyte, as a function of pH.

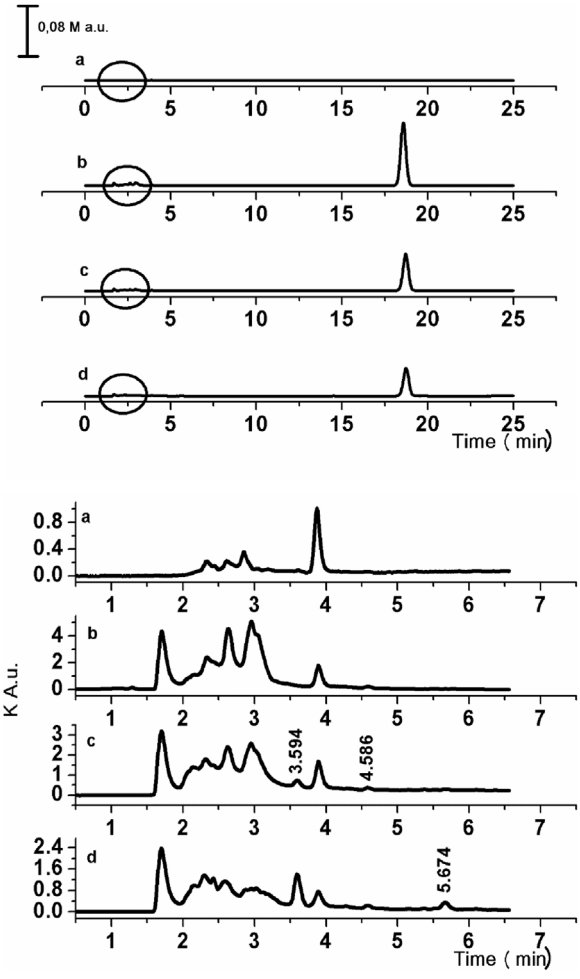


Fig. 5. HPLC chromatograms of 0.5 mmol L^{-1} CCNU solution in acetate buffer (pH 3.0), obtained using a PAD detector (λ =254 nm): (a) acetate buffer and ethanol; (b) freshly prepared solution; (c) after 24 h of incubation; (d) after 48 h of incubation.

while the two new peaks increased in intensity. In addition, another new peak with retention time of 5.674 min appeared, suggesting the formation of one more degradation product of CCNU in the aqueous medium.

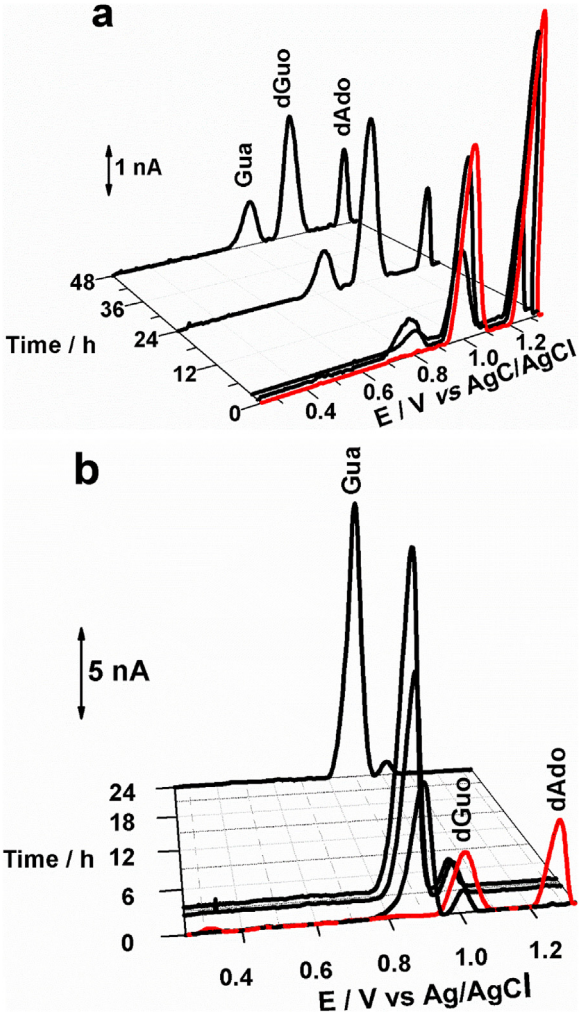


Fig. 6. Baseline-corrected DP voltammograms obtained in 0.1 M acetate buffer (pH 4.5): 100 μg mL^{-1} dsDNA control (▬) and incubated with: (a) 0.1 mmol L^{-1} CCNU during (▬) 1 h, 2.5 h, 24 h, and 48 h; (b) 0.1 mmol L^{-1} cdCCNU solution (14 d of degradation in pH 4.5 buffer) during (▬) 10 min, 2.5 h, 3.5 h, and 24 h.

3.3. *In situ evaluation of the interaction of CCNU with DNA*

The effects of the CCNU/dsDNA and cdCCNU/dsDNA interactions were evaluated by comparing the changes in the DNA oxidation peaks, dGuo and dAdo, in the absence and presence of these species in solution, and by monitoring the appearance of peaks corresponding to the oxidation products of guanine (8-oxoGua) and/or adenine (2,8-oxoAde), which occur at E_p = + 0.45 V in acetate buffer (pH 4.5) and would be indicative of oxidative DNA damage [25–27]. Evidence of a specific interaction between dGuo and dAdo residues was also evaluated electrochemically according to the appearance of peaks corresponding to free guanine (Gua), at E_p = + 0.85 V, and free adenine (Ade), at E_p = + 1.15 V, in acetate buffer solution (pH 4.5) [27]. The oxidation peaks of Gua and Ade arise from oxidation of the C1' carbon of deoxyribose, which results in the release of bases [25,26].

3.3.1. *In situ interaction of CCNU with dsDNA solutions*

The voltammogram recorded for the dsDNA control in pH 4.5 acetate buffer showed two anodic peaks, at E_p = + 1.01 V for dGuo and at E_p = + 1.27 V for dAdo (Fig. 6a). After 1 h of incubation, there were no significant changes of the dsDNA peaks, but a new peak appeared at E_p = + 0.83 V, corresponding to the oxidation of Gua. After 2.5 h of interaction, there were marked decreases in the cur-

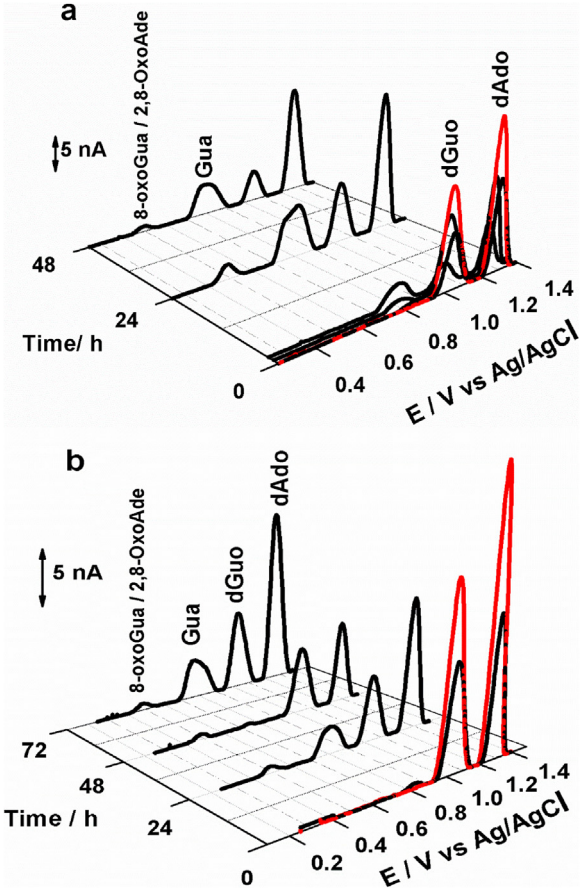


Fig. 7. Baseline-corrected DP voltammograms obtained in 0.1 M acetate buffer (pH 4.5): 100 μg mL^{-1} multilayer dsDNA biosensor control (▬) and incubated with: (a) 0.5 mmol L^{-1} CCNU during (▬) 10 min, 1 h, 2 h, 24 h, and 48 h; (b) 0.5 mmol L^{-1} cdCCNU solution (14 d of degradation in pH 4.5 buffer) during (▬) 10 min, 24 h, 48 h, and 72 h.

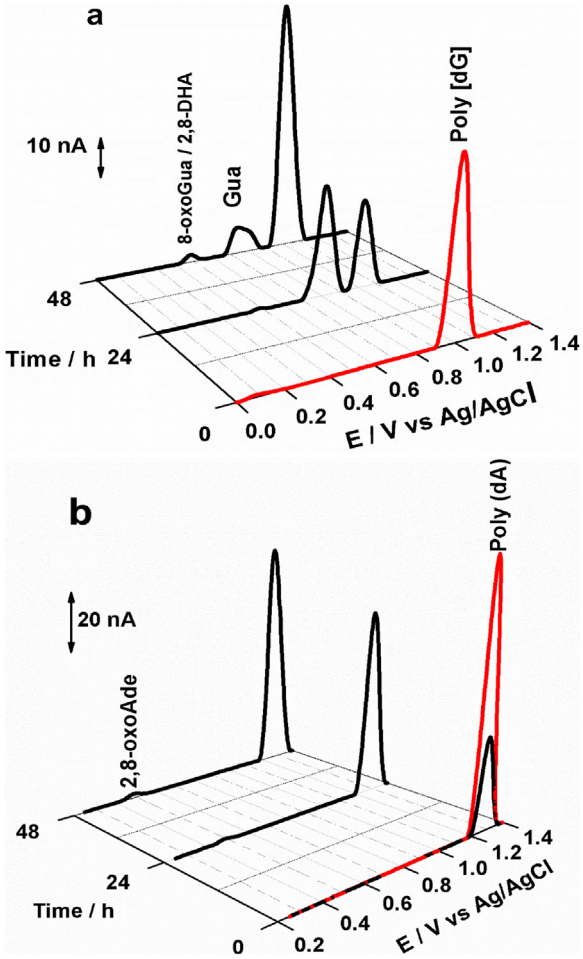


Fig. 8. Baseline-corrected DP voltammograms obtained in 0.1 M acetate buffer (pH 4.5): 100 μg mL^{-1} multilayer polyhomonucleotide biosensor control (▬) and incubated with 0.5 mmol L^{-1} cdCCNU (14 d of degradation in pH 4.5 buffer). (a) Poly(dG) incubated during (▬) 24 h and 48 h. (b) Poly(dA) incubated during (▬) 10 min, 24 h, and 48 h.

rent intensities of the nucleotide oxidation peaks, especially in the case of dAdo. This could have been due to the condensation of the double helix of dsDNA, which became compacted following interaction with the drug, making it difficult to expose it to the surface of the working electrode. After 24 h and 48 h, the dAdo peak current remained constant, while the Gua peak increased, due to its increased concentration in the solution. The peak current corresponding to the dGuo nucleotide increased after 24 h and 48 h of interaction, probably due to a change in dsDNA conformation, with unwinding of the helical chains resulting in a larger electrochemical signal [25].

In the cdCCNU/dsDNA interaction study, the dsDNA peaks were detected at E_p = + 1.00 V for dGuo and at E_p = + 1.27 V for dAdo (Fig. 6b). Immediately after adding the cdCCNU solution to the dsDNA solution, there was a significant decrease of the dGuo peak and disappearance of the dAdo peak, showing a greater interaction of cdCCNU with dAdo. This behavior indicated structural changes associated with condensation of the double helix chain, under the conditions employed. The Gua peak appeared at E_{pa} = + 0.88 V. Over the course of the incubation time, there was a successive decrease of the dGuo peak current, while there was a successive increase for Gua, due to the increase of its concentration in the solution.

The results showed that CCNU interacted with dsDNA and that its metabolite(s) caused a conformational change in the dsDNA strands, leading to a possible double helix condensation and greater interaction with the dAdo nucleotide, as well as release of bases from the double helix DNA.

3.3.2. *In situ interaction of CCNU with dsDNA biosensors*

In relation to the dsDNA/CCNU interaction (Fig. 7a), it was observed that during the first 10 min of incubation, the dsDNA peaks decreased and a new peak appeared at E_p = + 0.82 V, due to the oxidation of Gua in the solution. After 1 and 2 h of incubation, the dsDNA peaks decreased considerably and consequently the oxidation peak increased. For a longer incubation time (24 h), increases of the dsDNA peaks were observed, probably due to unwinding of the double-helix chain in solution, and the Gua concentration increased, since the current intensity at E_p = + 0.82 V increased. In addition, a peak appeared at E_p = + 0.48 V, indicating oxidative damage caused to the dsDNA by the pro-drug, due to the oxidation of 8-oxoGua and/or 2,8-oxoAde in acid solution. After 48 h of incubation of the dsDNA biosensor in the CCNU solution, the behavior of the pro-drug was the same, since all the peaks remained practically constant in relation to the current intensity.

Similar behavior was observed for the dsDNA/cdCCNU interaction (Fig. 7b), with a peak at E_p = + 0.48 V after 24 h indicating oxidative damage to the dsDNA, as discussed above. The peaks remained practically constant after 48 h of incubation, while after 72 h, the current intensities of the four detected peaks increased, due to the increased concentrations of 8-oxoGua and/or 2,8-oxoAde and Gua, together with the unwinding of the dsDNA strands in the solution. This unwinding probably led to the opening of the double

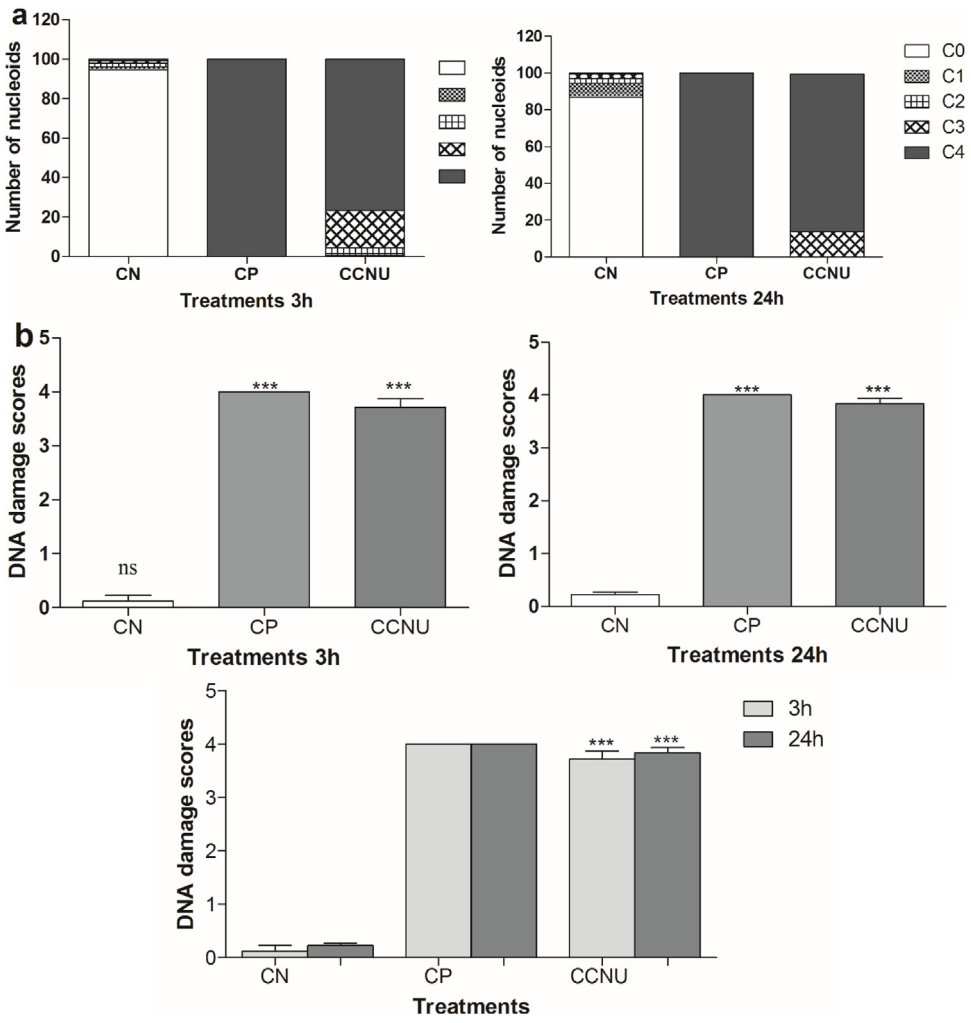


Fig. 9. (a) Frequency of comet classes and (b) DNA damage scores detected by comet assay using peripheral blood mononuclear cells (PBMCs) from healthy volunteers, 3 h and 24 h after treatment with 0.5 mmol L^{-1} CCNU in TAE buffer (pH 13). NC – negative control; PC – positive control, ***$p < 0.0001$; NS – not significant; C0 – class 0, C1 – class 1, C2 – class 2, C3 – class 3, C4 – class 4.

helix chain, with the consequent exposure of the bases facilitating their oxidation and the formation of the observed biomarkers [14].

3.3.3. *In situ interaction of cdCCNU with DNA using the poly(dG) and poly(dA) biosensors*

In order to obtain more information about the interaction and the oxidative damage caused to the dsDNA by the CCNU pro-drug metabolites, studies were performed using the poly(dG) and poly(dA) homopolymer-based biosensors with the cdCCNU solution.

After 24 h of incubation (Fig. 8a), the poly(dG) peak at E_p = + 1.00 V decreased considerably, compared to the peak for the control poly(dG) biosensor, which could be explained by condensation of the DNA strand morphological structure [28]. Additional peaks detected were at E_p = + 0.81 V, related to the oxidation of Gua, and a small peak at $E_p \approx$ + 0.48 V, related to the oxidation of 8-oxoGua [25–27]. After 48 h of interaction, the poly(dG) peak current increased, confirming the unwinding of the dsDNA in solution, and the peak of the biomarker increased, due to the increase of its concentration in the solution.

As shown in Fig. 8b, the behavior was similar to that observed in the previous study. After 10 min of interaction, the poly(dA) peak at E_p = + 1.26 V decreased considerably, compared to the control poly(dA) biosensor. However, this peak showed successive increases after 24 h and 48 h of incubation. In addition, a small peak at E_p = + 0.48 V, related to the oxidation of 2,8-oxoAde, was observed after 24 h and increased after 48 h of incubation.

3.3.4. *CCNU genotoxicity using comet assays*

Fig. 9 shows the results obtained in the alkaline comet tests, in which the nucleoids were classified according to the damage caused. The test was performed using two treatment times (3 h and 24 h). In the first 3 h of incubation with 0.5 mmol L^{-1} CCNU, there was a significant increase in the amount of DNA damage, compared to the negative control ($p < 0.0001$). After 24 h, only the presence of classes 3 and 4 was observed in the cells treated with CCNU (Fig. 9a), which represent more than 75% of DNA damage.

Application of the one-way ANOVA test showed that there was a significant increase in the damage scores obtained following exposure of the lymphocytes to CCNU for increasing times ($p < 0.0001$, Fig. 9b). These results confirmed that the compound caused direct DNA damage. According to the literature [29], the main component of the tail in the alkaline comet assay corresponds to the linear fragments of the single strand of DNA. This DNA damages confirm that the CCNU is a genotoxic agent, but DNA damage can be repaired by mechanism of DNA repair in the cells [30].

4. Conclusions

The electrochemical investigation of CCNU showed the compound underwent only reduction on the GCE, characterized by two irreversible processes that were diffusion-controlled, pH-dependent, and each one involved the transfer of two electrons and one proton. The degradation study showed that CCNU spontaneously degraded in aqueous solution, which was more accentuated in basic media. This instability was confirmed by chromatographic analysis.

The interaction study with DNA showed that CCNU and its metabolites interacted with dsDNA, causing conformational changes in the DNA strands, double helix condensation and subsequent unwinding, and breaking, as the incubation time increased. There was Gua release and oxidative DNA damage, as shown by the detection of 8-oxoGua and/or 2,8-oxoAde.

Therefore, the findings demonstrated that DNA electrochemical biosensors constitute a viable and highly accessible platform capable of providing information about the interactions of antineoplastic compounds with DNA structures, contributing to the development of new anticancer drugs that are more effective and selective.

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